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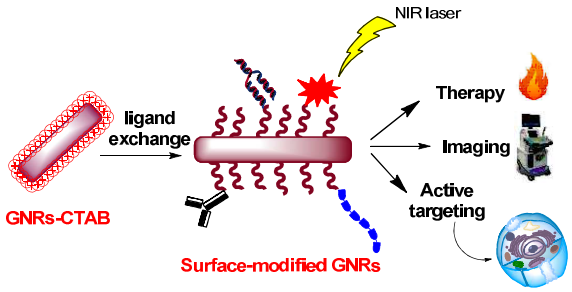

Surface modification of Gold Nanorods allows biocompatibility and complex architecture design for novel theranostic possibilities.

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REVIEW

Surface Modifications of Gold Nanorods for Applications in Nanomedicine.

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Gold nanorods (GNRs) are appealing nanostructures for a wide variety of nanomedicine-based diagnostic and therapeutic approaches against untreatable diseases. Indeed, they possess unique and extraordinary optical features, which if conveniently stressed, would bring several benefits to non-invasive theranostic treatments. Major concerns regarding their real employment derived, not directly from GNRs, but from molecules linked onto their surface, which could be source of toxicity as well as powerful allied for treatments' enhancement. Thus, specific and tailored surface modification of GNRs with several active moieties has become a crucial point for their development. In this review, steps forward and major possibilities deriving from GNRs surface decoration for final nanomedicine applications will be summarized and discussed, as well as progress in therapy and diagnosis relying on functionalization of these nanosystems.

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REVIEW

1. Introduction

Unlike other metal nanostructures, gold nanorods (GNRs), have appeared fascinating in many scientific field, among which nanomedicine is certainly one of the most important.

The success that GNRs for medical purpose is partially related to the fact that gold has been demonstrated a highly biocompatible material, since it presents very low toxicity even at high concentrations, no damage to organs after prolonged exposition, and excretion via the hepatobiliary system.[1,2] Moreover, their longitudinal plasmon resonance (LPR) can be finely "moved" toward high wavelengths up to 1200 nm by simply increasing their aspect ratio (ratio between length and width): in this way it can fall in the range 800-1200 nm (**Figure 1**) where is placed the so-called Near Infrared (NIR) Window, particularly attractive for medical applications due to the high transmittance (low absorbance) of water, deoxygenated haemoglobin and oxygenated haemoglobin, which allows the use of laser without interfering with- or burning healthy tissues and organs.[3]

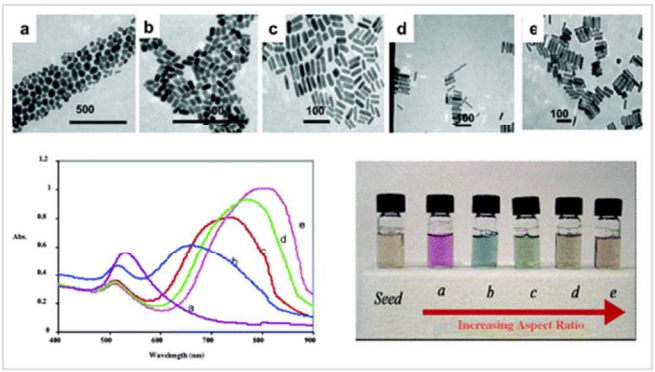

Figure 1: GNRs optical properties variation by increasing their aspect ratio. From *From* C. J. Murphy *et al., J. Phys. Chem.,* 2005, **109**, 13857.

GNRs represent an innovative contrast agent for non-invasive diagnostic techniques such as optoacoustic imaging and X-ray computed tomography, because they present considerable advantages in comparison to other common contrast agents as molecular dyes, fluorophores or quantum dots which all present poor stability, photo bleaching under common imaging conditions, high toxicity and frequently insufficient absorption cross section and scattering signal.[4]

In addition, GNRs has been investigated also as therapeutic tool since more than 96% of the absorbed radiation is converted into heat due to the higher absorption cross section of GNRs than of other nanostructures: this means that GNRs absorb light rapidly but also that the relaxation process is slower and the result is an energy release in form of heat. The generated local hyperthermia could easily reach several degrees.[5] A so strong localized increment in temperature can be exploited to selectively destruction of cancer cells or diseased tissues under laser irradiation as a powerful alternative to medical surgery or invasive therapies, making GNRs a real and appealing therapeutic agent.

Due to these reasons, GNRs are nowadays finding applications in nanomedicine as the most promising theranostic (therapeutic + diagnostic) agent.

Despite the attractive possibilities opened up by GNRs, their synthesis and surface's modification still represent a limit for wider applications. Mostly, the synthesis of GNRs occurs in aqueous medium with the assistance of various surfactants, which both act as template and stabilize the growing nanoparticles against aggregation phenomena.[6] These surfactants remain adsorbed or deposited onto the nanoparticles surface once the process is finished, avoiding post-synthesis collapse of the created nanoparticles. Unfortunately, most of these surfactants are strongly toxic or simply not suitable for the desired final application since they do not allow further synthetic modifications.

The removal of surfactants requires the development of specific ligands able to replace them, to prevent the aggregation phenomena, and at the same time to ensure the specific desired final properties to GNRs.

In this review, after a due paragraph concerning the synthesis of GNRs, the possibilities of surface modification with several moieties will be covered: organic molecules, synthetic polymers, natural biopolymers, peptides or proteins, oligonucleotides, DNA and RNA all have been exploited for the coating of GNRs and their subsequent application in nanomedicine. Finally, the most significant successes derived from surface-modified GNRs in the field of theranostic will be discussed.

1. Synthesis of GNRs

Nowadays, even if several methodologies ranging from chemistry to physics have been exploited for the preparation of GNRs with different aspect ratio, they remain mostly synthesized by using the so-called "seed-mediated growth method", which allows reproducible results and an easily tunable final aspect ratio. This methodology was firstly optimized for the synthesis of GNRs by Nikoobakht and El-Sayed in 2003.[7] In a general procedure, a high concentration of the surfactant, cationic agent cetyltrimethylammonium bromide

(CTAB) is used in order to permit GNRs formation and simultaneously to avoid GNRs aggregation and precipitation once synthesized.

The method consists in preparing "seeds", which can work as nucleation sites, reducing a small amount of tetrachloroauric acid ($HAuCl_4$) in aqueous solution and in the presence of the surfactant with a strong reducing agent such as sodium borohydride ($NaBH_4$); a small amount of these seeds is then introduced in the real growth solution, containing Ag^+ ions, an excess of the surfactant and a more abundant amount of $HAuCl_4$ already partially reduced from Au^{3+} to Au^{1+} by ascorbic acid in order to facilitate the deposition of gold onto seeds during the growth stage. All the reaction occurs in 24 hours, at room temperature (around 30°C) and in aqueous environment, thus allowing its application also in common, and not particularly equipped laboratories.

Several studies confirmed that CTAB is arranged in a double layer (bilayer) around GNRs in the growth phase: first layer present CTAB's head-group oriented towards the NPs surface while in the second layer is directed towards water, leaving into the centre the two hydrophobic tails, in a fashion close to cellular membrane.[8]

Positively charged silver ions seems to be intercalated between the negatively charged bromine head groups on the surface of the nanostructure, thus limiting the tendency of the negative charged heads to repel each other, and promoting the elongation of GNRs: in fact it has been showed that, within certain limits, the greater the amount of silver ions present in solution, the greater the aspect ratio of GNRs obtained, thus making very easy to tune size or shape of the GNRs therefore controlling their properties.

The fact that the growth occurs preferentially in one direction rather than in all the possible ones is attributed to the role of CTAB, which during the nucleation step creates a preliminary facial differentiation of the seeds. Once immersed in the growth solution, the seeds undergoes the attack of the surfactant preferentially on the more accessible face {100}, while the face remained free from CTAB {111} can grow, thus leading to the elongated cylindrical structure. For the same reason, the {111} face remains in each stage more reactive than the {100}, a fact that will influence all the surface chemistry of these nanostructures (**Figure 2**).[9]

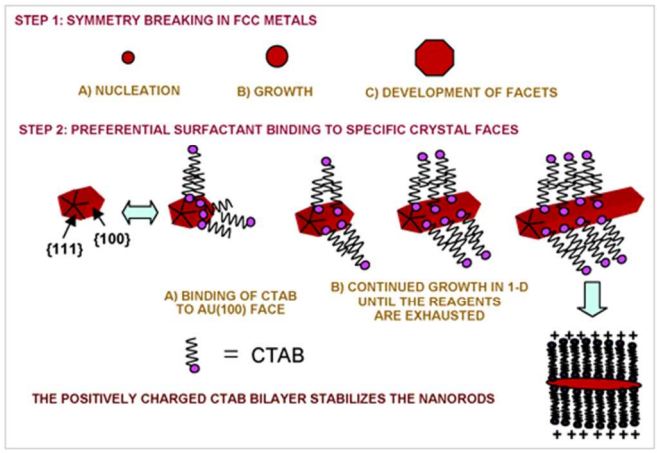

Figure 2: Formation mechanism of GNRs. *From* C. J. Murphy *et al., J. Phys. Chem.*, 2005, **109**, 13857.

It has been demonstrated that free CTAB molecules, desorbing from GNRs surface once in physiological conditions, have a strong cytotoxic effect on healthy cells,[4] due to its ionic nature, which leads to a strong interaction with DNA and RNA molecules and, in addition,, their complete removal from GNRs surface always gives an immediate irreversible aggregation. CTAB replacement seems to be the only alternative but it represents a great challenge due to the different reactivity of the {111} and {100} nanorods' faces.[10]

3. Surface modification of GNRs

In the last decade many efforts were done to address this issue, especially by developing a plethora of molecules or moieties that can substitute toxic surfactants in their role and simultaneously allow, reinforce or modify GNRs theranostic properties; moreover several protocols assisting this replacements have been described in literature and many others are entering into the scientific landscape, highlighting the urgent need for a review summarizing the various options currently available **(Figure 3)**.

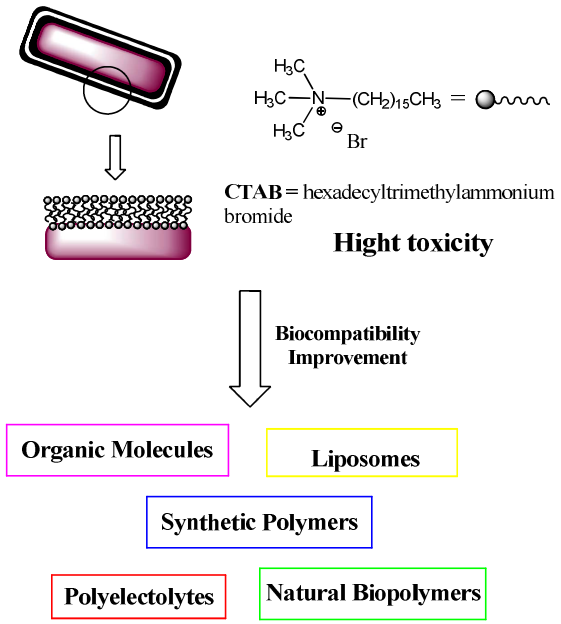

Figure 3: Surface modification of GNRs

3.1 Organic molecules

In recent years many studies reported in the literature have showed the possibility to remove CTAB from GNRs surface and replace it with various organic molecules.

In some cases organic molecules have been directly linked to GNRs surface, exploiting the presence of functional groups particularly affine to gold. These molecules can bind gold surface leading to the formation of a self-assembled monolayer (SAM) and to the displacement of the double layer of CTAB (**Figure 4**); in other cases, organic molecules are previously modified by binding with other molecules such as a linker, which constitute the real coating layer on the surface of GNRs.

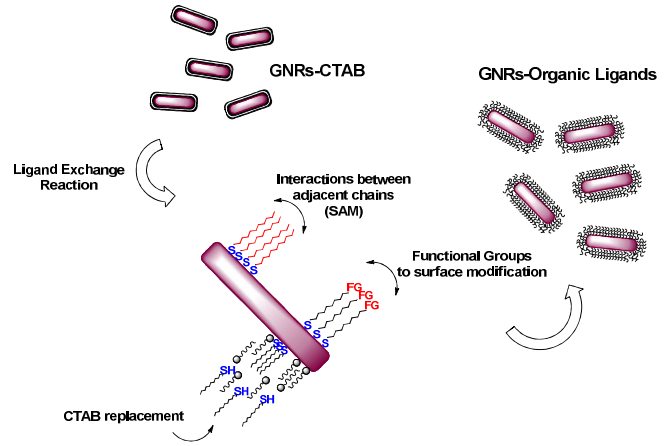

Figure 4: example of SAM onto the surface of GNRs.

Thiol group is the most used functional group to bind gold surface. Indeed, sulphur is characterized by a great affinity to the transition and noble metals.[11] For this reason, thiol groups can bind gold surfaces strongly to form a self-asslembled monolayer. In this way, it is possible to form an organic protective layer on GNRs surface by replacement of CTAB and to obtain GNRs with a greater stability, a better biocompatibility and hydrophilic or lipophilic properties, according to organic molecules that are used.

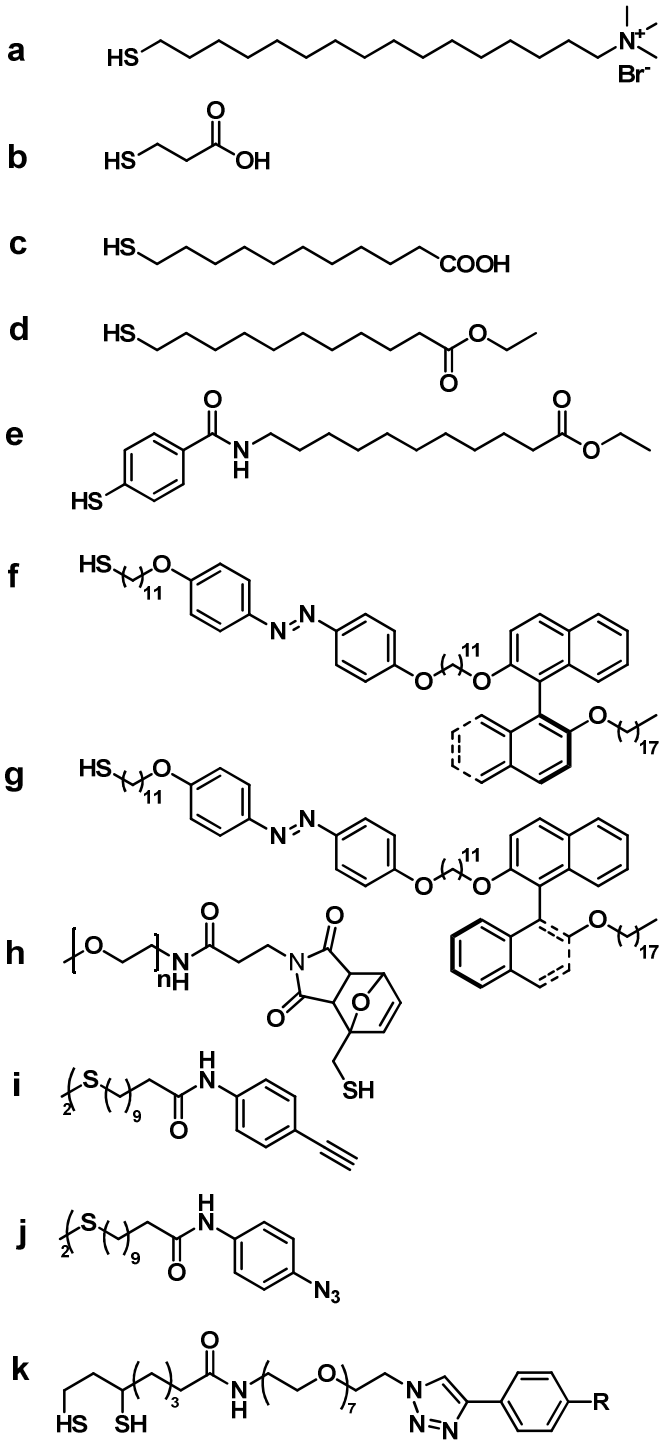

Figure 5: examples of thiol-based ligands used for GNRs coating.

Thiolated CTAB and different thiol ligands to modify GNRs surface have been used in ligand exchange reaction also. In particular Vigderman *et al.*[12] reported a strategy to coat GNRs with the thiolated CTAB analogue (16-mercaptohexadecyl)trimethylammonium bromide (MTAB) (**Figure 5a**). The MTAB ligand contains an entire CTAB moiety and a pendant thiol group which can be used to anchor the molecule on gold surface. In this way a compact monolayer on the GNRs surface is formed.

In their study, Garabagiu *et al.*[13] reported the ligand exchange reaction with 3-mercaptopropionic acid (**Figure 5b**). In this case 3-mercaptopropionic acid can be used as linker thanks to the presence of carboxylic group that could be used for any further functionalization. On the other hand Dai *et al.*[14] showed that it was possible to obtain GNRs soluble in both polar and non-polar organic solvents. In this case the authors used 11-mercaptoundecanoic acid (MUA) (**Figure 5c**) in a ligand exchange reaction through ion exchange resin to obtain GNRs-MUA stable and soluble in both chloroform and methanol.

However, in the ligand exchange reaction on GNRs surface not all organic thiol ligands could be used. We described lipophilic GNRs synthesis by ligand exchange reactions using two different organic thiols: ethyl 11-mercaptoundecanoate and ethyl 11- (4-mercaptobenzamido) undecanoate (**Figure 5d-e**).[15] The study showed that the exchange reaction and the consequent formation of lipophilic GNRs occur only with ethyl 11- (4-mercaptobenzamido) undecanoate. The different behaviour could be explained by the different structural proprieties of the two ligands which confer different robustness and stability to the obtained GNRs. The presence of aromatic and hydrogen-bonding moieties, such as amides, allow adjacent molecules (ligands) to form multiple interactions providing the formation of stable and robust monostrate on GNRs surface.

Usually the use of organic molecules is not limited to coat GNRs surfaces and to improve biocompatibility. In some cases, specific organic molecules have been used to give specific chemical properties to GNRs. An example is the study reported by Li *et al.*[16] who describes the coating of GNRs with two opposite chiral azo thiol enantiomers synthesized starting from (R)-(+)-1,1'-bi-(2-naphthol) (**Figure 5f**) and (S)-(-)-1,1'-bi-(2-naphthol) (**Figure 5g**). After surface modification with the two different enantiomers of chiral thiol, a protective organic monolayer on the surface is formed and the obtained GNRs acquired lipophilic and optical properties.

Moreover, GNRs can be used also to impart specific properties to other materials. For example, Ori *et al.*[17] used the thiol ligand ethyl 11- (4-mercaptobenzamido) undecanoate (**Figure 5e**) to form lipophilic GNRs for functionalization of a glass surface in order to constitute a powerful tool in areas ranging from electronics to biosensors. This system has been achieved by the immobilization of lipophilic GNRs on thiol functionalized glass surface and can confer optical properties to modified solid support.

When the organic thiol bear an additional functional group at the end of the alkylic chain several intersting features can be exploited. Yamashita *et al.*,[18] described the synthesis of GNRs coated with PEG-linked Diels Alder cycloadduct (**Figure 5h**) in which organic molecules anchored on the surface become substrates of chemical processes. In this reaction, GNRs are modified by insertion on the surface of cycloadducts via Au-S linkages. The so obtained GNRs are irradiated by NIR light which generate a photothermal effect inducing a retro Diels Alder reaction that releases PEG chains bound to the cycloadducts.

Another example of this type is represented by click chemistry reaction on GNRs surface coated by thiol organic ligands characterized by acetylene groups. We have described the development of a novel nanosystem consisting of GNRs assembled to silver nanoparticles, which presents both therapeutic and diagnostic capabilities.[19] Lipophilic GNRs were synthesized using simultaneously two ω-functionalized-disulfides characterized by the presence of esters and acetylenes, while for silver nanoparticles a terminal azide was chosen (**Figure 5i-j**). In this way it was possible to covalently link the two nanostructures, reacting through 1,3-dipolar click cycloaddiction between GNRs acetylenic and azide group on silver nanoparticles (**Figure 6**).

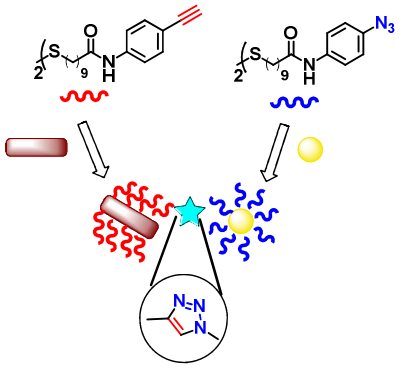

Figure 6: click chemistry between functionalized GNRs and silver nanoparticles.

However, polyethylene glycol thiolate (PEG-SH) is the organic thiol most used in ligand exchange reaction with GNRs, thanks to the well-known biocompatibility of PEG substrate. The use of synthetic polymers for these purposes will be discussed extensively in the next section. We briefly describe here the use of thiol-PEG as binding ligand on GNRs for deposition of other functional molecules. Dreaden et al.[20] published a study describing a novel delivery system to tumor associated macrophages (TAM). In this case macrolides, a class of antibodies for the treatment of microbial infections, were firstly covalently linked via click chemistry to thiol-PEG chains (**Figure 5k**) and then thiol groups of PEG are used to bind GNRs surface.

The thiol-PEG has been used as linker also by Son *et al.*[21] to immobilize mannose on gold's surface of gold/nickel nanorod to obtain nanobrigdes for immune cell recognition.

The coating with organic thiols is not the only way to remove CTAB and modify GNRs surface. Huang *et al.*[22] described GNRs coating with silica using APTES, a reagent commonly used to form SiO_2-NH_2 in core-shell systems. The layer of

silica is applied with Strober method to remove the CTAB and improve the biocompatibility of the obtained GNRs; also the presence of amino groups on the surface provides the possibility to functionalize the surface covalently conjugating targeting agents, such as in this case the folic acid.

3.2 Synthetic Polymers

Different synthetic polymers have been used to improve GNRs biocompatibility but especially PEG is the most utilized in the lieterature. It is well-known that PEG gives biocompatibility, stealth characteristics and resistance to protein adsorption providing a long plasma circulation time.

In particular, the study reported in literature by Grabinski *et al*.[23] related to an in-depth analysis on the toxicity comparing organic molecules and polymer coating surface of GNRs; two different nanosystems were synthesized from GNRs-CTAB by ligand exchange reaction using two organic molecules: mercaptoesadecanoic acid (MHDA) and thiol-PEG. The toxicity of these different systems has been investigated by the study of consequent gene expression in the cell lines. The *in vitro* studies showed that GNRs-MHDA have a dramatic effect on gene expression more than PEG-GNRs. This phenomenon could be explained because of their enhanced interaction with cell membranes compared to PEG-GNRs, which also led to greater uptake.

Recently, studies showed the use of not only "already formed" synthetic polymers but also different approaches to coat GNRs depending on the polymer used.

In this view, studies describing the synthesis of polymeric GNRs via radical polymerization are the most promising. Hotchkiss *et al*.[24] used reversible Addiction-Fragmentation Chain Transfer Polymerization (RAFT) to coat GNRs with synthetic polymers. The authors investigated three different polymers such as PDMAEMA (poly (2- (dimethylamino) ethyl methacrylate), PAA (poly (acrylic acid) and PS (polystyrene) and tested two different methods for the surface coating. In each case, they obtained GNRs completely surrounded by a relatively high polymer layer, the thickness of which depending on the polymer and grafting technique used.

On the other hand the study of Song *et al*.[25] reported the first example of surface-initiated living ring opening polymerization (ROP) of biodegradable polymers on GNRs to obtain nanosystems for plasmonic theranostic applications. They described the synthesis of amphiphilic GNRs coated with polylactid acid (PLA), via surface initiated organocatalytic living ring-opening polymerization (ROP), and PEG, via ligand exchange reaction.In the presence of water, the so-obtained GNRs formed well-defined vesicles consisting of PEG corona and GNRs-embedded PLA shell, characterized by unique combination of structural and optical properties.

Another interesting polymeric material used for GNRs coating are the dendrimers, a class of polymers with highly ordered branched structure. Dendrimers coating are used to modify the surface because of their capability to alter charge surface, functionality, and reactivity, as well as to enhance the stability and dispersion of the nanosystems. Li *et al*.[26] showed the use of polyamidoamine (PAMAM) thiolated dendrimer to synthesize dendrimer-modified GNRs, removing CTAB from GNRs surface and improving their stability and biocompatibility. Indeed, dendritic nanocomposites are characterized by different properties such as increased functional groups, symmetry perfection, and internal cavities, which make them excellent tools for applications in nanomedicine. In addition, dendrimer-modified GNRs were modified on the surface with a targeting agent, which confer high selectivity to the synthesized nanosystems.

As shown by these examples (**Figure 7**) in most cases synthetic polymers are used to coat GNRs surface by directly linkages of end-functionalized polymers on the surface through ligand exchange reaction. However, the synthetic polymers can also be used to form polymeric nanocarriers to entrap lipophilic GNRs and to form targetable biocompatible nanosystems. We[15,27] showed the synthesis of polymeric nanoparticles, made up of the amphiphilic copolymer poly(lactic-*co*-glycolic)-*co*-poly(ethylene glycol) (PLGA-*b*-PEG-COOH). This polymer consists by both PEG and a low-molecular-weight hydrophobic core-forming block, such as PLGA. Using this copolymer is possible to form GNRs containing polymeric nanopartilces, presenting a hydrophobic core to entrap lipophilic GNRs and a hydrophilic shell to allow nanosystem stabilization in aqueous solution as well as further surface conjugation reaction.

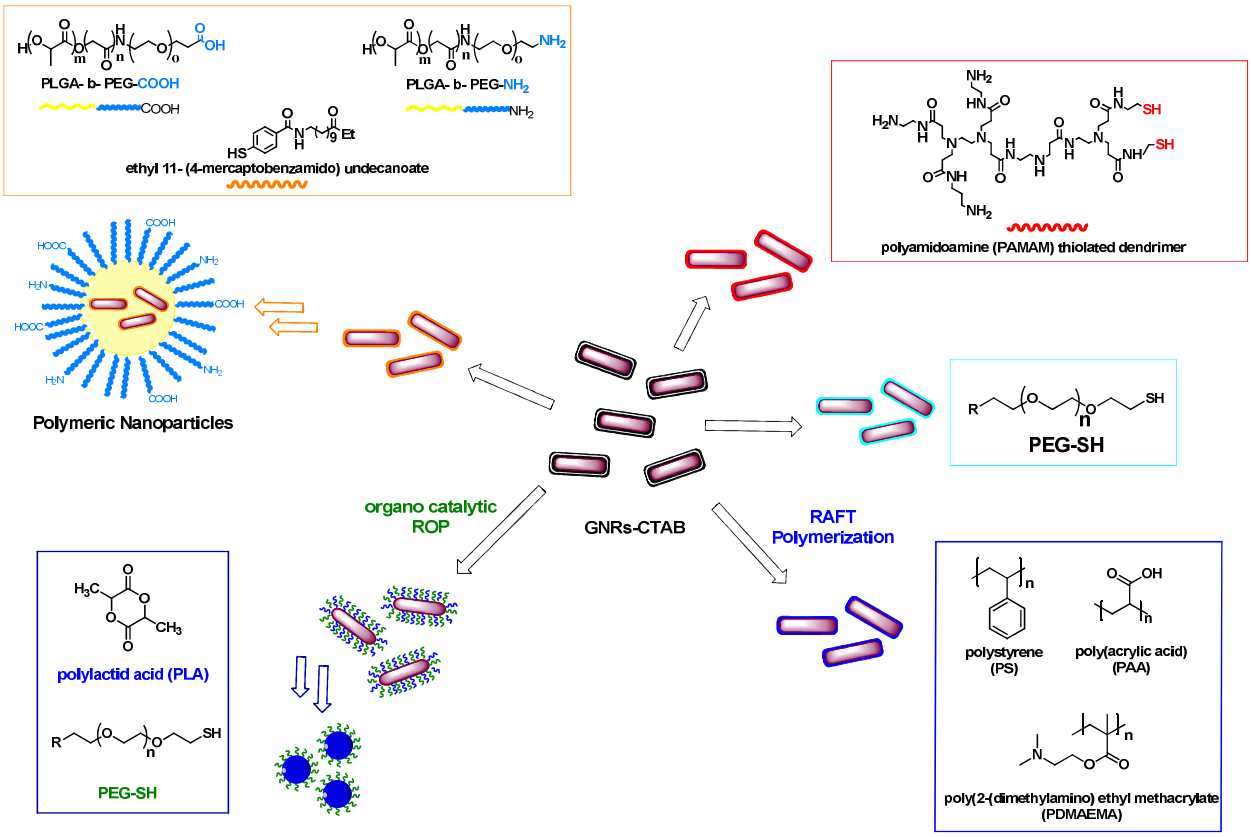

Figure 7: GNRs coating strategies by using synthetic polymers.

3.3 Polyelectrolytes

Widely used method of nanosystems coating is represented by electrostatic physisorption polyelectrolyte (**Figure 8**) to improve dispersion stability and to provide support for immobilization of targeted agents (antibiotics and proteins). However, nowadays it is still necessary to improve the stability and biocompatibility of polyelectrolyted nanosystems, which result generally cytotoxic. Indeed physisorbed polyelectrolytes, under certain physiological conditions, are easily desorbed by nanosystems surface because of their variable and labile surface binding energies.

One of the first examples regarding the use of polyelectrolytes to modify the surface of GNRs is reported by Gole *et al*.[28] The authors used the layer-by-layer method to form on positively charged GNRs-CTAB a polyelectrolyte multilayers by sequential deposition of anionic and cationic polyelectrolytes. At the beginning GNRs-CTAB are coated with the anionic poly (sodium-4-styrenesulfonate) (PSS) and then with the cationic poly(diallyldimethylammonium chloride) (PDADMAC). This process has been continued until a polyelectrolyte multilayers was formed on GNRs surface. In the study reported by Parab *et al*.[29] the same method has been used to coat GNRs-CTAB with poly (sodium-4-styrenesulfonate) (PSS). PSS modification allowed conjugating the IgG antibody on GNRs surface to investigate their cytotoxicity, cellular uptake and physiological detection of proteins. This study showed that PSS significantly increases cell viability and internalization of GNRs and also the presence of the antibody promotes the assembly of GNRs with preferential orientation in a lateral way (side-to-side and end-to-end).

However, polyelectrolyte multilayers prepared with the layer-by-layer method are characterized by a not well-defined interface between the various layers and also by non-covalent bond between organic moieties and the surface. These features confer instability to those modified nanosystems. Leonov *et al*.[30] discussed deeply the importance of CTAB replacement and developed a novel scalable protocol for surfactant exchange based on polyelectrolytes-coated GNRs. However, in this work it was shown that PSS-coated GNRs are characterized by high cytotoxicity due to the presence of CTAB complex-PSS which are gradually desorbed from the surface of GNRs and it is therefore necessary a careful purification to obtain the complete replacement of CTAB. In addition it has been demonstrated that cellular uptake is strongly influenced by different superficial charge that characterized the synthetized polyelectrolytes-GNRs. The studies reported by Hauck at al.[31,32] assessed the relationship between surface coating and cellular uptake. GNRs have been covered by layer-by-layer method with various polyelectrolytes, giving nanorods characterized by different surface charge. The study showed that the lowest cellular uptake was exhibited by negative surface charged GNRs, while

the highest cellular uptake was exhibited by the positively charged system.

The same concept has been reported by Xu *et al*.[33] The study described the use of GNRs as possible vehicle for gene delivery, in particular as DNA vaccine adjuvants. Two different cationic molecules, Poly (diallyldimethyl ammonium chloride) (PDDAC) and Polyethyleneimine (PEI), have been used to modified GNRs surface on which HIV Env plasmid DNA was conjugated. Biological assays shows that PDDAC- and PEI modified GNRs can significantly improve cellular and tumoral immunity due to the surface chemistry on the adjuvant activity.

Another interesting method for GNRs surface modification involves the use of phospholipids, which form a double surface layer on the surface. The study by Takahashi *et al*.[34] described phospholipids-GNRs formation by extraction using a chloroform phase. They showed that CTAB is successfully removed from GNRs solution by simple extraction using chloroform containing phosphatidylcholine (PC), as additional stabilizing agent. Indeed, PC is a possible candidate for suppressing GNRs aggregation after CTAB extraction and for reducing cytotoxicity.

Finally Orendoff *et al*.[35] developed a method to synthesize phospholipids-GNRs by using the lipid vesicle fusion approach. In this way it was possible to obtain GNRs with phopholipid vesicles consisted of phosphatidylcholines lipids by immobilizing liposomes at the nanorods surface. Although this polyelectrolyte differed from CTAB surfactant in net electrostatic charge at neutral pH, both are terminated with trimethylammonium head groups that may interact similarly with GNRs surface. This work showed the possibility to modify GNRs surface with different functional groups by coating with single component ligand or bilayers.

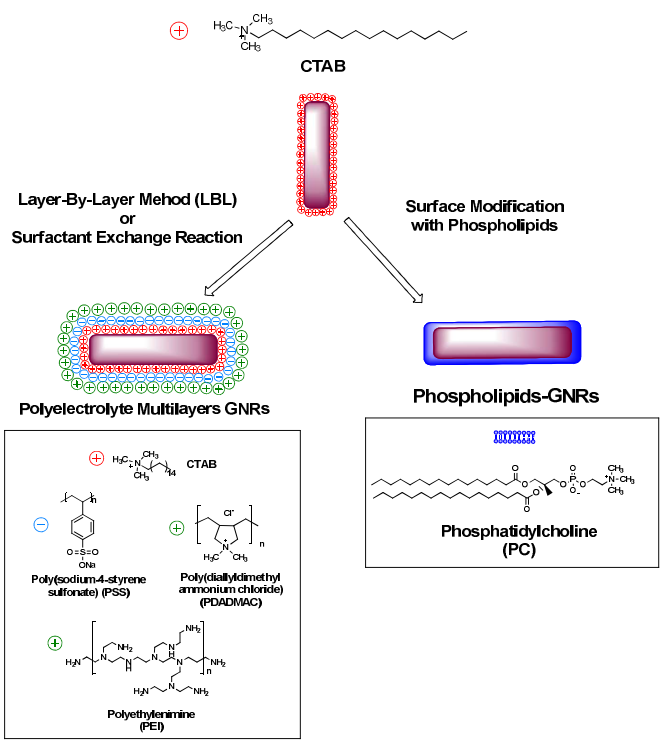

Figure 8: : GNRs coating strategies by using polyelectrolytes and phospholipids.

3.4 Peptides, proteins and natural sugars

Natural materials, such as natural polysaccharides, natural polymers, peptides and natural molecules, have been used extensively as possible materials in the development of biocompatibility drug delivery systems. Indeed, they have important features such as high stability, biodegradability, biocompatibility that make them potential tools for biomedicine applications.

Chitosan is a non-toxic, biocompatible, biodegradable and natural polysaccharide, which is produced by deacetylation of chitin, contained in the exoskeleton of some crustaceans and insects. This natural polysaccharide is used successfully in the nanomedicine applications for the delivery of drugs, gene and protein. In particular, also GNRs have been coated with chitosan by using different techniques and materials in order to develop a novel biocompatible theranostic nanosystem. Indeed, chitosan can be covalently bound to GNRs surface in different ways. It is possible to covalently bind chitosan to organic molecules presented on their surface, or also to chemically modify chitosan chains with organic molecules characterized by functional groups able to coordinate GNRs surface (thiols groups). In both cases, the formation of covalent bonds involves a coupling reaction to form a stable amide bond between carboxylic group of organic molecules, activated in some cases by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) chemistry, and amine groups present over the surface of chitosan.

In the study reported by Charan *et al*.[36] chitosan oligosaccharide-modified gold nanorods were synthesized by using of 11-mercaptoundecanoic acid (MUA). Chitosan have

been linked carboxylic groups of MUA on GNRs. The study showed that chitosan-MUA-GNRs exhibited the least toxicity in compare to the other synthetized surface-modified gold nanorods. The resulting chitosan-GNRs were conjugated with tumor targeting monoclonal antibody against EGFR which showed good cellular uptake and biodistribution.

Even in the work of Garabagiu *et al*.,[37] GNRs have been coated with chitosan through a cross-linker, the 3-mercaptopropionic acid (MPA). The chitosan-MPA-GNRs are synthetized without using of EDC Chemistry but left the reaction under magnetic stirring for 2 days.

Organic molecules utilized to modify chitosan are characterized by features able to improve chitosan by chemical properties and functional groups able to coordinate gold's surface. In the study of Wang *et al*.[38] chitosan was covalently grafted with mercaptoacetic acid (MAA). The obtained modified chitosan-MAA are characterized by the presence of thiol groups that bind GNRs surface. In addiction the targeting agent folic acid has been conjugated on GNRs surface.

R. Duan *et al*.[39] in their work have modified chitosan with polyethilene glycol (PEG) and thiolated polyethylenimine (PEI). The introduction of these hydrophilic polymer chains enhanced water solubility of chitosan, which normally has a good solubility only in acid conditions (pH<5). Following the doxorubicin (DOX) was chemically conjugated on thiol modified chitosan and the as-synthetetized chitosan-polymer-DOX was used to coat GNRs to obtain novel nanocarriers with good biocompatibility and optical properties.

On the other hand Choi *et al*.[40] have synthesized nanocarriers for GRNs by using photopolymerization with chitosan chemically conjugated to Pluronic F68. The obtained GNRs showed increased cellular uptake *in vitro* and a photothermal effect for cancer cell line, suggesting a promising feature for clinical phototerapeutic applications.

Also peptides and other natural materials have been used to modify GNRs surface in order to improve cellular uptake. For example, the study of Khan *et al*.[41] GNRs have been coated with four different amphiphilic ligands (LA) in order to study how different ligands properties could influence protein crown formation and consequently cellular uptake. Three different amphiphilic ligand by ligand exchange reaction from GNRs-CTAB have been synthesized: neutral charged GNRS-polyoxyethylene (10) cetyl ether characterized, cationic GNRs-phospholipid oligofectamine and anionic GNRs IPID-phosphatidylserine (PS). The study showed that the protein crowns formation and their physical properties was influenced by the nature of the amphiphilic ligands.

In the study of Murakami at al.[42] GNRs have been modified with natural molecules, in particular (Z) -9-Octadecenoate (oleate) and high density lipoprotein (HDL) (a mediator of reverse cholesterol transport), which can interact with tissues and cells. The results showed that the as-synthesized cpHD-GNRs were internalized greater than 80 times more efficiently than poly- (ethylene glycol) -conjugated GNRs and were able to elicit cancer cell photoablation.

In other exemples the GNRs surface has been decorated with different peptide which may act as targeting agents such as in study reported by Alkilani *et al*.[43] They have functionalized GNRs with the EphA2 homing peptide, YSA, using a layer-by layer polypeptide wrapping approach. The peptide was linked to a polyelectrolyte chain (polyaspartate) via a PEG linker, in order to enable polyelectrolyte wrapping around the starting cationic GNRs. The obtained peptide-functionalized GNRs have been used to explore how the presence and orientation of the YSA peptide can influence GNRs stability on biological media, cellular uptake, and proliferation in cancer cells.

Also Park *et al*.[44] have described a method to functionalize GNRs with an engineered fusion protein in which gold-binding polypeptide (GBP) was fused with Staphylococcal Protein A (SpA). The replacement of free CTAB during the functionalization step prevents CTAB-induced aggregation of the GNRs and GBP-SpA complexes. The resulting nanosystems can be easily functionalized and conjugated to form a potential tool for theranostic applications.

Finally, in the study of Jang *et al*.[45] a multifunctional nanomedicine platform consisted of gold nanorods-photosentizer complex has been developed for non-invasive near-infrared fluorescence imaging and cancer therapy. The synthesis of this nanosystem consist of sequentially conjugation on GNRs surface of thiol-terminated monomethoxy poly(ethylene glycol) (mPEG-SH) and a targeting ligand consisting of a positively charged oligo peptide made of arginine (R), leucine (L) and cysteine (C) (RRLAC). Then the negatively charged ptotosensitizer Au(III) phthalocyanine chloride tetrasulfonic acid ($AlPcS_4$) was incorporated onto positively charge PEG-GNRs-RRLAC. This paper showed the possibility to combine a photosensitzer agent with the GNRs to combine photothermal therapy (PTT) with photodynamic therapy (PDT) in cancer treatments.

3.5 Monoclonal antibodies

Monoclonal antibodies are widely recognized as one of the most selective and promising agent to target cancer cells. They are engineered to exploit their natural ability to recognize only a single specific antigen, making them potentially suitable for active drug delivery of nanoparticles against any disease.[46] Moreover monoclonal antibody have advanced significantly over the past two decades also as therapeutics for cancer therapy, showing the appealing possibility to act both as targeting agent and as therapeutic selective drug.[47]

It is clear that their linking onto GNRs could lead to important advantages thanks to the merge of such important features with the characteristic of those nanostructures.

Firstly in 2006, El-Sayed's research was focused on this topic.[48] In this study an anti-epidermal growth factor receptor (anti-EGFR) monoclonal antibody was conjugated onto the surface of GNRs previously capped with poly(styrenesulfonate) through electrostatic physisorption interactions: the aim was to demonstrate the efficacy of GNRs *in vitro* onto malignant oral epithelial cell lines as contrast agent for both molecular imaging and photothermal cancer therapy. Thanks to the

antibody GNRs were able to selectively bind the malignant cells with a much higher affinity due to the overexpressed EGFR on their cytoplasmatic membrane. In this case only electrostatic bond for the interaction between GNRs and monoclonal antibody was exploited. Another example of GNRs modified with monoclonal antibodies is found in the work of Park *et al*.[49] who published in 2009 the layer-by-layer deposition of anti-rabbit IgGs onto the surface of GNRs by electrostatic interactions in order to enhance the targeting and imaging of biomarkers expressed on the surface membrane of cancer cells. In this case mercaptopyridine was attached to the surface of GNRs and their surface charge was modified using a layer of poly-(sodium 4-styrene-sulfonate) switching it from positive to negative in order to enable antibody electrostatic absorption.

More recently, Choi *et al*.[50] covalently linked GNRs and cetuximab, an anti-epidermal growth factor receptor, in a two-steps reaction: firstly GNRs were coated with a hetero-bifunctional PEG, bearing both a thiol and a carboxylic acid group (HS-PEG-COOH), thus removing CTAB molecules and increasing biocompatibility, then the carboxylic groups remained exposed onto the surface were exploited for the covalently linkage with an amino group present on cetuximab internal structure. The so-obtained functionalized GNRs showed excellent tumor targeting ability and promising effect in imaging and photothermal therapy of epithelial cancer cells.

Similarly, Liao *et al*.[51] and Puvanakrishnan *et al*.[52] covalently conjugated monoclonal antibody onto GNRs surface exploiting a mixed coating with methoxy-PEG-thiol and thiol-PEG-thiol in order to dilute the reactive groups onto GNRs' surface, thus avoiding cross-linking issues. The remaining terminal thiol group at one end of the bifunctional PEG was reacted with the antibody previously functionalized with a maleimide-containing crosslinker agent.

Despite the improvement in GNRs efficacy that can be achieved with antibodies decoration, many approaches still suffer of several downsides including low stability and potential cytotoxicity of bioconjugates that are produced by electrostatic interactions, as well as lack of control over antibody orientation during covalent conjugation. Due to this reason, Joshi *et al*.[53] investigated a "directional" antibody conjugation onto GNRs' surface. In their work the directional conjugation was achieved by oxidizing the carbohydrate moiety, presents specifically on the heavy chain of the Fragment crystallizable (Fc) region of most antibodies, to an aldehyde group and then by attaching it with a hetero-functional linker with hydrazide and dithiol groups on the GNRs' surface. The obtained modified GNRs were tested both for stability and cancer cells recognition with satisfactory results.

3.6 Oligonucleotides

Gene therapy has been studied intensively during the last years due to its appealing features. Using RNA, DNA or chemically-modified oligonucleotides the overcoming of protein-based treatments issues, such as immunogenic *in vivo,* lack of thermal stability and large scale synthesis, became possible. Nucleic acids-based moieties can be divided in several categories basing on their composition and structure (single strand oligonucleotides, antisense oligonucleotides, DNA decoy, RNA decoy etc…) or their target (proteins, genes, micro RNA etc…).[54] Oligonucleotides have shown promising ability both as therapeutic agents, since they can interact in cells gene-expression and life cycle, and as targeting species due to their almost unique specificity against a target receptor.[55,56]

Despite the already available pool of papers concerning nanoparticles modification with oligonucleotide,[57] there are still few examples of such kind of modification onto GNRs.

One of the first examples of oligonucleotide-conjugated GNRs for nanomedicine applications has been reported only in 2005 by Takahashi *et al*.,[58] who employed phosphatidylcholine modified GNRs for plasmid DNA anchoring and release under NIR stimuli. Later on, in 2006 Chen *et al*[59] reported the first preparation of a DNA-fragment covalently attached onto GNRs via a thiol group, previously inserted in the DNA sequence. In this case, DNA was selectively released upon ultrafast NIR laser irradiation from GNRs in order to induce specific gene expression in target cells. In 2008, Wijaya *et al*. exploited a similar procedure[60] for the conjugation and selective release of two different DNA oligonucleotides from two gold nanorods with different aspect ratio just trigging laser wavelength. A particularly appealing innovation was proposed by Xu *et al*.,[33] who investigated novel surface-engineered GNRs used as promising carrier for DNA vaccine against HIV disease: three different kinds of molecules were placed onto GNRs (CTAB, poly(diallydimethylammonium) chloride and polyethyleneimine), and their transfection capability, internalization, cellular trafficking and DNA releasing were all related to the primary surface modification, thus casting light on the rational design of nanomaterials as a versatile platform for vaccine adjuvants/delivery systems. Recently, Shanmugam *et al*.[61] successfully developed a hybridized double stranded DNA, ending with a thiol group for conjugation on GNRs, which worked as intercalating binding site for Doxorubicin and as tethering agent for platinum [Pt(IV)] prodrugs; in this case cancer cells were targeted with folic acid covalently conjugated to the acid group in the axial ligand of platinum pro-drug. This complex architecture could release the two chemotherapeutics under NIR laser exposure as result of GNRs generated hyperthermia, thus limiting toxicity of common cancer treatments.

Small interfering RNA (siRNA) appeared coupled with GNRs in 2009 in the work of Bonoiu *et al*.,[62] who developed GNRs-siRNA complexes (called nanoplexes) that target the dopaminergic signalling pathway in the brain. In particular GNRs were used to show the effective interaction with siRNA trough shifts in their longitudinal plasmon resonance and to visualize neurons *in vitro*. From the same authors is also a patent released in 2011 regarding methods of using GNRs-siRNA complexes for gene therapy.[63] One last example of GNRs-siRNA complex could be found in the work of Tahmasebifara *et al*.[64] who investigated the various phenomena occurring during GNR-siRNA nanoplexes formation. The

authors, using several analytical methodologies, gave an important insight into the nature of the interaction between metal surface and such biomolecules.

Aptamers, another class of potent and very modern oligonucleotides, were also exploited in conjugation with GNRs. Indeed, in 2012[65] and with improvements in 2013,[66] Wang *et al*. interestingly developed an aptamer switch probe linked to a photosensitizer molecule and covalently attached to the surface of GNRs, able to modify its conformation, thus releasing the photosensitizer molecule, only in presence of target cancer cells for combining photodynamic therapy (PDT) and photothermal therapy (PTT). A different aptamer was used by the same group to target and kill cancer cells and cancer steam cells upon GNRs photothermal activation.[67]

This overview suggests that with time more interest has been posing in oligonucleotides-conjugated GNRs and an increasing number of publications appeared in scientific journals, even if the total amount remains scarce and calls for more studies.

Table 1: summary of viability and cytotoxicity tests performed on surface modified GNRs

Entry	Surface Modification	Test	Cell Line/ Tumour	Results	Ref.
1	SiO_2-NH_2/Folic Acid	CCK-8 assay	MGC803 Cells	GNRs-SiO2-FA possesses non-cytotoxicity and excellent biocompatibility.	22
2	Mercaptoesadecanoic Acid (MHDA) and Thiol-PEG	MTS Test	HaCaT (Keratinoicyte Cells)	Ligand exchange to MHDA and PEG resulted in improved viability and lower toxicity.	23
3	Vesicles consisting of PEG corona and GNRs-embedded PLA shell	Combined Dual-Modality Chemo-Photothermal Therapy with CCK-8 assay	*In vitro:* Hep 3B cells	GNR@PEG/PLA vescicles showed no toxicity 24 h after uptake by cells.	25
4	Dendrimer-modified GNRs (dGNRs)	Cytotoxicity studies with Kit-8 assay and selective photothermal therapy of dGNRs	*In vitro:* HUVEC (non-malignant cells) A375 (melanoma cell lines)	RGD-coniugated gGNRs are not cytotoxic.	26
5	GNRs-(11- (4-mercaptobenzamido) undecanoate) entrapped in polymeric nanopartilces	Cytotoxicity studies with CFE assay.	*In vitro:* Balb/3T3	IC_{50} = 20.3 μM	27
6	poly (sodium-4-styrenesulfonate) (PSS)	Cytotoxicity and Cellular Uptake Studies with MTS assay.	*In vitro:* S-G (normal gingival epithelioid cells) TW 2.6 (Oral cancer cells)	PSS significantly increased the cell viability and showed easy intracellular uptake of the nanorods	29
7	poly (sodium-4-styrenesulfonate) (PSS)	Cytotoxicity studies with MTT and LDH assays.	*In vitro:* LLC-PK1 (porcine kidney cells) HepG2 (human liver carcinoma cells) KB (human nasopharyngeal carcinoma cells)	Cytotoxicity assays of PSS-coated GNRs revealed IC_{50} values in the low to submicromolar range.	30
8	Different charged polyelectrolytes (layer-by-layer method)	Cell Uptake and transmission electron microscopy (TEM);	In vitro: OCI AML3 (myeloid leukemia cells) Jurkat T-cells MCF-7 (breast cancer	The study showed that the lowest cellular uptake was exhibited by negative surface charged GNRs, while the highest cellular uptake was	31, 32

			cells)	exhibited by the positively charged system	
9	Phosphatidylcholine (PC) (phospholipids-GNRs)	Studies of cytotoxicity	I*n vitro:* HeLa cells	GNRs-PC show lower cytotoxicity than GNRs-CTAB.	34
10	Chitosan-MUA (11-mercaptoundecanoic acid)	Cytotoxicity studies with MTT Assay and study of Toxicity *in vivo.*	*In vitro:* CAL 27 (carcinoma cells) *In vivo:* BALB/c nude mice model	Chitosan modification exhibited low toxicity and rapid excretion. .	36
11	Chitosan-mercaptoacetic acid (MAA)	Cytotoxicity studies with MTT Assay.	*In Vitro:* HT-29 (colorectal carcinoma cells) NHI 3T3 (embryonic fibroblast)	Cell viability of CTAB-passivated GNRs was improved by thiolated chitosan capped GNRs.	38
12	Chitosan - polyethilene glycol (PEG)/thiolated polyethylenimine (PEI)/doxorubicin (DOX)	Cytotoxicity test CCK-8 assay.	*In vitro:* MCF-7 (breast cancer cells) A549 (lung cancer cells) HeLa (cervical cancer cells) and L929 (fibroblast cells)	Chitosan modified GNRs have been demonstrated to have good biocompatibility and stability.	39
13	Chitosan-Pluronic F68	Cytotoxicity test and cellular uptake.	*In vitro:* SCC7 (tumor cells) NIH/3T3 (fibroblast cells)	Chitosan conjugated GNRs have been shown any acute cytotoxicity.	40
14	YSA, EphA2 homing peptide	Cytotoxicity studies with MTS assay	*In vitro:* PC-3 Cells	Functionalized GNRs induced no acute cytotoxicity under the concentrations tested.	43
15	PSS and anti-EGFR monoclonal antibody	Light scattering images	*in vitro:* non-malignant epithelial cell line (HaCat) and two malignant oral epithelial cell lines (HOC 313 clone 8 and HSC 3)	no cytotoxicity evidence after 30 minutes incubation	48
16	mercaptopyridine, poly-(sodium 4-styrene-sulfonate) and anti-rabbit IgGs	dark-field images	MCF7 breast cancer cells.	no cytotoxicity evidence after 2 hours incubation	49
17	COOH-PEG-SH and cetuximab	MTT assay	A-431 and MCF7 cell line.	10-fold lower cytotoxicity after 24 hours incubation for conjugated GNRs in	50

				comparison with non-conjugated GNRs	
18	methoxy-PEG-thiol and thiolated antibody.	MTS assay	A431, MDAMB-435 cancer cells and J774A.1 macrophage cells.	no cytotoxicity of surface modified-GNRs. Strong cytotoxicity for CTAB coated GNRs	53
19	pEGFP-N1template DNA-SH	trypan blue assay	HeLa cells	no induced cytotoxicity. After NIR exposure gene expression was revealed after 1-2 day only for GNRs antibody-conjugated	59
20	double stranded DNA with doxorubicin or platinum prodrugs plus folic acid.	MTT assay	HeLa cells	Enhanced toxicity and drug release under NIR exposure of surface modified GNRs in comparision to components alone	61
21	siRNA (nanoplexes)	MTT assay	DAN cells	98% viability one week post treatment	62
22	mPEG-SH, ASP and Ce6 photosensitizer	MTS assay	CCRF-CEM cells	cell viability decreased to about 80% after combined PTT and PDT therapy.	65-66
23	CSC1 aptamer and CSC13 aptamer	MTS assay	DU145 cells	strong cytotoxicity after exposure to aptamer-conjugated GNRs and NIR irradiation	67

4. Nanomedicine applications of GNRs

As already said GNRs' great success during the last years is most of all related to their wide exploitable and tunable properties for nanomedicine applications. The abovementioned features of GNRs make them suitable as contrast agent for early diseases' diagnosis as well as therapy mediator, allowing in both cases the use of non-invasive techniques. Indeed the possibility to combine together diagnosis and therapy is at the base of the innovative field of Theranostic (therapeutic + diagnostic), which is now worldwide leading the most innovative researches (**Figure 9**).[68]

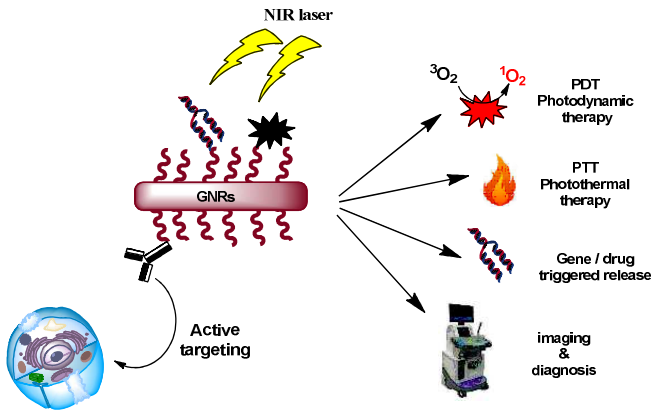

Figure 9: Theranostic approaches deriving from surface-decorated GNRs

GNRs strong light absorption and scattering allow their use in several imaging techniques able to lead to an early pre-symptomatic diagnosis of various diseases, such as dark-field microscopy,[69,70] optical coherence tomography (OCT),[71,72,73] two-photon luminescence (TPL),[74,75] photoacoustic (PA),[27,76] X-ray computed tomography (CT) imaging[77] and ultrasound (US).[78] For what concern the therapy, GNRs great ability to convert light into heat is mostly exploited through the hyperthermia effect for photothermal therapy (PTT)[79,80,81] but also to trigger thermo-sensitive release of active moieties in drug delivery systems;[60,82] recently, as mentioned earlier, GNRs-photosensitezer composites have been developed and exploited in photodynamic therapy (PDT).[66]

Development and improvement of an appropriate coating of GNRs have speeded up, and in some cases made possible, those researches. Since various reviews already satisfactory described all the appealing possibilities to use GNRs in nanomedicine, both for diagnosis and for therapy, the next paragraphs will focus mostly on the progress made in these research fields thanks to the possibility to decorate GNRs in a fashionable manner with several organic molecules and active components.

4.1 Imaging with surface-modified GNRs.

When speaking of imaging techniques assisted by the use of GNRs it must be distinguished between *in vitro* imaging and *in vivo* applicable imaging. Dark field microscopy and two-photon luminescence are mostly related to a cellular environment while optical coherence tomography, photoacoustic imaging and X –

ray computed tomography are already applicable pre-clinically and to living being.[4]

Dark field microscopy is widely used to localize GNRs in the cellular environmental and, for example, to demonstrate the selective targeting of these nanostructures against specific target sites when coated with selective active moieties such as proteins, peptides, monoclonal antibodies and oligonucleotides, as already discussed above. A perfect example of this technique, which is at the base of the first papers concerning GNRs imaging, is represented by the work of El-Sayed,[48] who not only investigated the variation in the different colored scattered light by changing GNRs aspect ratio, but also showed that GNRs anti EGFR-conjugated scattered differently after binding to the malignant or nonmalignant cells: indeed, while cancerous cells showed a red to orange scattered light, individual noncancerous cells are hardly identifiable due to the non-specific interactions between the nanoparticles and the cells. Following this research several other investigations, both practical and theoretical, have appeared among the scientific literature.[83,84,85]

Two-photon luminescence using GNRs followed a similar pathway: indeed it was observed that when molecules are adsorbed on the surface of noble metal particles, a huge field enhancement occurs, which gives rise to the well-known surface enhanced Raman scattering (SERS) signals of molecules but also to a spectrally broad background photoluminescence (PL).[86] Even if studies of PL from metal nanoparticles were limited, due to their low quantum efficiencies, strong enhancement of PL from GNRs upon single photon excitation was reported, probably due to their unique SPR.[87] Two-photon optical processes, which involve an additional field enhancement, and thus, a greater enhancement of PL efficiency, has been recently applied for GNRs imaging.[88] As well as for dark-field microscopy, also TPL has been mostly exploited for cancer cells detection *in vitro*, after binding with targeting agents-conjugated GNRs. Durr *et al*.[75] in 2007, demonstrated with phantom experiments that the TPL intensity from GNRs-labeled cancer cells was 3 orders of magnitude brighter than the two-photon auto-fluorescence (TPAF) emission intensity from unlabeled cancer cells at 760 nm excitation light, showing that GNRs can be an attractive contrast agent for two-photon imaging of epithelial cancer. More recently the first application of TPL in *in vivo* systems has been also reported. Wang *et al*.[89] demonstrated the possibility to use GNRs as TPL imaging agents by *in vivo* monitoring of single nanorods flowing in mouse ear blood vessels, thanks to the intrinsic 3D spatial resolution of TPL, which can be useful for monitoring biological processes in real time even if it lack of deep tissues penetration. Unfortunately in this work no particular attention was posed in GNRs functionalization, as they were maintained coated with the original CTAB molecules, but the results achieved is worth of mention, and surely appropriate coating will help improving this imaging modality.

More appealing possibilities derived from the use of well-establish imaging techniques, already suitable for living system. Optical coherence tomography (OCT), an interferometric imaging technique based on the detection of backscattered NIR light, can provide an innovative, non-invasive diagnostic methodology due to good penetration depth as well as high spatial resolution, which are both superior to the one of present clinical methods of non-invasive imaging, such as ultrasound or magnetic resonance imaging. The use of enhancer contrast agents for this technique is strongly encouraged, and GNRs have been recently discovered to be excellent nanostructures for this task.[90] GNRs have been shown to produce a detectable OCT signal at the minimal concentration of 25 μg Au/ml in intralipid suspension. Moreover, Troutman *et al*.,[90] showed that GNRs with plasmon resonance wavelengths overlapping the OCT source yielded a signal-to-background ratio of 4.5 dB in tissue phantoms. Despite the great potentiality of this methodology still few papers appeared to report an *in vivo* diagnostic application. One of the first attempt has been done by Tucker-Schwartz *et al*.,[91] who applied photothermal - optical coherence tomography (PT-OCT) for *in vivo* imaging using GNRs as contrast agent: *in vivo* PT-OCT images were acquired after subcutaneous injection into mice of 400 pM GNRs embedded in a gel matrix and revealed an appreciable increase in signal in the presence of GNRs compared to controls, demonstrating the possibile translation of PT-OCT from *in vitro* to *in vivo* imaging.

Probably the most used modality to visualize GNRs is photoacoustic imaging (also known as optoacoustic imaging). The intrinsic conversion of light into heat in GNRs is exploited in this technique, which involve the use of laser pulse irradiation and the detection of the resulting acoustic waves generated from the temperature gradient and expansion of illuminated contrast agent. The technique itself already found large applications for tumor detection[92] or blood oxygenation monitoring,[93] but GNRs can provide an increasing detection capability, thus amplifying the field's application.

Agarwal *et al*.,[94] showed that GNRs, with peak absorption in the range of 700-840 nm, conjugated with an antibody designed specifically for the Her-2/neu antigen, overexpressed in LNCaP prostate cancer lines, can be used to enhance optical absorption and photoacoustic signals in targeted prostate cancer tissue, thus providing high contrast for non-invasive cancer imaging of a single layer of cells. Meanwhile, Li *et al*.,[95] demonstrated the possibility to exploit different aspect ratio GNRs for the combined PA imaging and measuring of the expression levels of different oncogenes in cancer cells simultaneously: interesting, in this study an antibody for HEGR2 antigen, expressed in MBT2 (murine bladder cancer) cells and a another one for CXCR4 antigen, expressed in HepG2 (human hepatocellular carcinoma) were used as target molecules. These two monoclonal antibodies were conjugated to the surface of two types of GNRs with different aspect ratios (5.9 and 3.7) and different optical absorption peaks (1000 and 785 nm, respectively). Appropriate selection of laser irradiation wavelength allows PA signals only from GNRs corresponding to specific bindings, thus making them discernable.

In vivo applications of GNRs as PA contrast agent have been also reported since 2007. Eghtedari *et al*.,[96] investigated the detection limit of GNRs *in vivo* with PA: for this aim GNRs were coated with mPEG-thiol chains or alternatively with PSS, in order to render them suitable for *in vivo* injection. The results showed that 25 µL of GNRs at a concentration of 1.25 pM can be detected in mice after subcutaneous injection using a single-channel acoustic transducer, confirming that these nanostructures are powerful contrast agents for PA imaging. More recently, we[27] demonstrated the possibility to detect suface-modified GNRs entrapped in biodegradable polymeric micelles highly suitable for advanced drug delivery applications: as already explained, with a double phase transfer protocol, lipophilic GNRs can be encapsulated into physiologically stable, biocompatible and targetable micelles, meanwhile remaining detectable with PA imaging till the concentration of 11 µM. Latterly, Li *et al*.,[97] continued their studies regarding discernable cancer cells by using different aspect ratio GNRs and demonstrated the same possibility also *in vivo*. In addition, in this case two different GNRs were coated with two monoclonal antibodies and with PEG in order to use them *in vivo* and to avoid non-specific interaction between antibodies and GNRs, then they were injected in subcutaneously tumors bearing mice. The results clearly showed presence of GNRs at the tumor site, and increasing in PA signals only when the corresponding antibody was conjugated onto the surface, allowing not only easily tumor detection but also the possibility to distinguish different cancer types.

On top of the most applied clinical imaging techniques, ultrasound (US) present unique features in terms of low cost, manageability, non-invasively and real-time imaging. Even if US are already widely used by clinicians, the sensitivity of this diagnostic method could greatly improve by exploiting ultrasound contrast agents (UCA).[78] GNRs, especially when entrapped into soft-materials such as polymeric capsules or micro-bubbles have been demonstrated to aptly serve the scope. In one of our paper we demonstrated[27] in phantom study the possibility to visualize GNRs previously entrapped into polymeric micelles using only US imaging: gel spheres at concentration in GNRs ranging from 550 µM to 11 µM, were imaged with a single laser pulse delivering an energy of approximately 10 mJ cm^{-2} and were clearly detected by US.

This technique was recently applied also *in vivo* by Ke *et al*.,[98] who deposited through electrostatic interaction GNRs modified with PSS onto the surface of microcapsules made of polylactic acid and modified with a layer of poly(allylamine hydrochloride) (PAH); the so obtained nanosystem was evaluated for acoustic enhancement: *in vivo* imaging of the kidney of rabbits post injection provided a clear and detailed view of renal vascularity, with excellent enhancement compared to the same investigation without contrast agent.

However, since the same instrumentation is used both for US and PA techniques, frequently they are coupled to create dual methodologies that provide complementary information. This combination came under the name of photoacoustic ultrasound (PAUS) imaging.[99]

Wang *et al*.[100] prepared cystamin's modified GNRs in order to increase biocompatibility and to enable their entrapment into human serum albumin microbubbles (MB), which can be detected both from US and PA imaging. Dual-modality contrast enhancement obtained with PAUS was demonstrated into polyacrylic acid gel spheres loaded with the hybrid nanosystem, and the enhancement obtained in signal and resolution was evident (**Figure 10**).

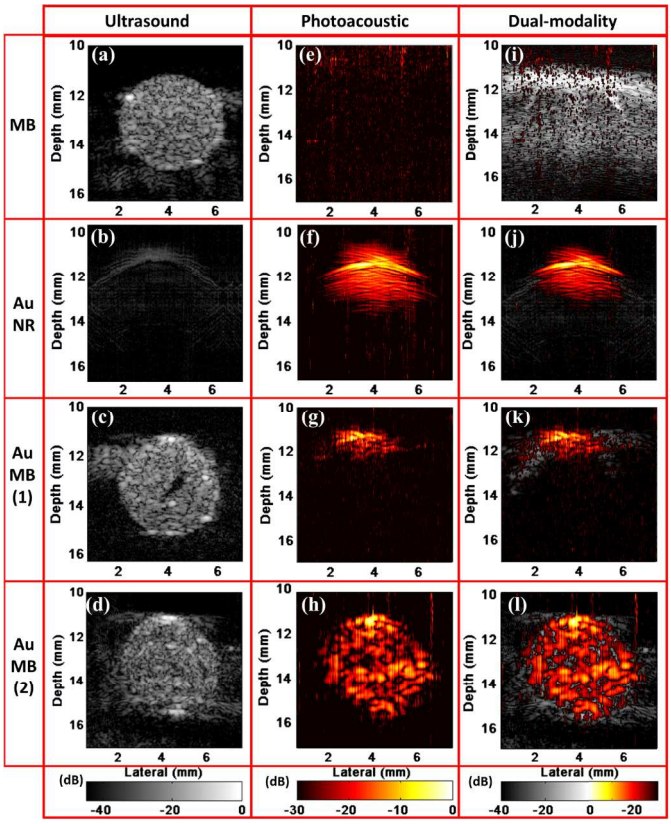

Figure 10: (a-d) US images of MB, AuNR, 1% AuMB, and 50% AuMB; (e-h) PA images of MB, AuNR, 75% AuMB, and 50% AuMB; and (i-l) fused images of 100% MB, AuNR, 75% AuMB, and 50% AuMB, where the US and PA data are in gray-scale and thermal color, respectively. From Wang *et al*. *Biomed. Optics*, 2012, **17**, 045001.

In vivo application of PAUS has also been developed. For instance Kim *et al*.[101] produced biocompatible GNRs, coating their surface with mPEG-thiol polymer and using it as a silane coupling agent for silica coating. The silica-coated GNRs were chosen due to their enhanced thermal stability and photoacoustic signal response. Ultrasound-guided photoacoustic imaging in xenograft tumor bearing mouse, was exploited to image the tumor and to guide the therapy as well as for monitoring temperature raising during the treatment. After intravenous injection of silica-coated GNRs and sufficient circulation time, nanoparticles delivery and spatial distribution was evaluated with three-dimensional PAUS, then PTT was performed: during PTT, photoacoustic images were acquired

continuously and used to measure the temperature changes within tissue.

X-ray computed tomography being one of the most exploited diagnostic techniques in hospitals nowadays has been considered as powerful instrument for molecular imaging. Due to their high atomic weight and strong X-ray absorption, GNRs have been evaluated as novel contrast agent for this technique, also because iodine, the most involved contrast agent today, present severe side effects, such as kidney toxicity, and fast body excretion. Moreover, the possibility to guide GNRs toward the desired site of action taking advantage from the surface conjugation of targeting molecules represents another appealing strategy for a most effective diagnosis.

Since most of the basic investigations were already carried out on gold nanoparticles, GNRs were directly evaluated for advanced studies, such as the one reported by Luo *et al*,[102] who proposed silica coated- indocyanine green embedded- GNRs for a dual-mode X-ray CT and NIR fluorescence imaging: 12 hours post intratumoral injection of 200 µL of GNRs coated solution at concentration of 1.5 mg/mL, X-ray CT scanning showed that this system could provide significant contrast enhancement, while NIR fluorescence generated by the dye was still present, thus enabling its use as a promising dual mode imaging contrast agent. Equally Huang *et al*,[22] developed a folic acid conjugated silica-modified GNRs for X-ray CT and photothermal therapy, testing them *in vivo* and showing promising results in a perfect example of real theranostic (**Figure 11**).

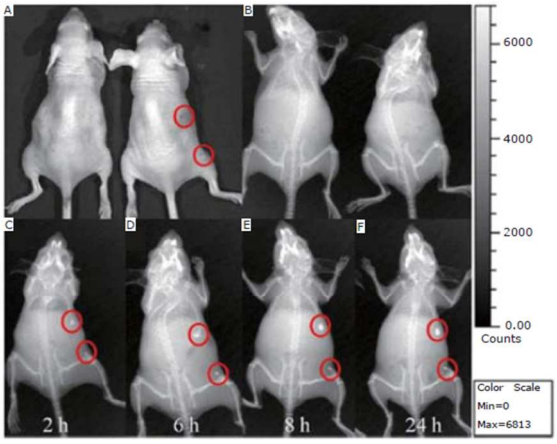

Figure 11: *In vivo* X-ray imaging of mice after subcutaneous injection (left) without and (right) with GNR-SiO_2-FA at different time points. (A) The photograph of mice; (B) the X-ray image at 0 h; (C) the X-ray image at 2 h; (D) the X-ray image at 6 h, (E) the X-ray image at 8 h; (F) the X-ray image at 24 h. From Huang *et al*., *Biomaterials*, 2011, **32**, 9796.

4.2 Therapy and theranostic with surface-modified GNRs

As already said most of therapeutic strategies relying in GNRs can be remotely controlled by NIR light which can penetrate deep into human tissues with minimal collateral effects.

Among these strategies the most widely investigated treatment has been those based on hyperthermia, generated during the so-called photothermal therapy (PTT) gaining advantages by the powerful conversion of light into heat that takes place in GNRs. Due to the longitudinal plasmon resonance falling in the NIR window, where the interaction between light and tissues is minimal, GNRs have been elected among all the other nanoparticles to be employed for this application.[103] Even if the local temperature increase could reach hundreds of degrees by using GNRs, such an extreme temperature is often not requested for *in vivo* burning and destruction of malignant cells, thus also the power of the laser could be reduced to more tolerable values.[104]

Several papers appeared when searching the literature for keywords such as "GNRs photothermal therapy" therefore only innovative together with those, which clearly show an advantage by the surface modification of GNRs will be summarized in this review.

For what concern *in vitro* results, mechanism of the induced damages by hyperthermia in malignant cells, which bring to cells death, has been thoroughly investigated, bringing to the conclusion that the disruption of the cytoplasmic membrane and the entrance into cell compartment of large amount of Ca^{2+} ions from plasma as a consequence of the GNRs heating during laser exposure is the primary cause of cells death.[105] Similarly, Cabada *et al*., demonstrated the effectiveness of continuous wave laser irradiation of GNRs in glioblastoma cell lines, analyzing the mechanism at the base of the photothermal cells death, reaching the same conclusion reported above.[106]

Surface-targeting of GNRs for selective malignant cells destruction have also been tested: Chlorotoxin-targeted polymeric micelles-entrapped GNRs has been reported by us and showed glioblastoma cells death, while the not targeted analogs showed very low toxicity after laser exposure, confirming the necessity to an effective targeting of these nanostructures into cells for a better efficacy.[80] Equally, GNRs conjugated with both the targeting agent transferrin and PEG, were used for combining TPL and PTT onto HeLa cells: while TPL allowed imaging of GNRs-transferrin incubated cells, no uptake was observed when the targeting agent was not present; equally, clear cell death was observed only when GNRs-transferrin were used, with a laser power of 25 mW after 20 scans (1.05 s per scan).[107]

Concerning application *in vivo* of this technique, Dickerson *et al*. reported the use of PEG-modified GNRs, with prolonged blood circulation, for *in vivo* PTT, after both intratumoral and intravenous injection in subcutaneous tumor bearing mice, obtaining significant results in term of tumor reduction: an inhibition of average tumor growth for both delivery methods over a 13-day period was observed, with a specific tumor re-absorption of >57% of the directly-injected tumors and 25% of the intravenously-treated tumors.[81] Li *et al*.,[26] improved PTT with GNRs by developing thiolated polyamidoamine (PAMAM) dendrimers as replacement for the CTAB molecules onto the surface of GNRs and by conjugating an arginine-glycine-aspartic acid (RGD) peptides, for selective targeting of the melanoma A375 cell line with overexpression of $\alpha_v\beta_3$. The so-obtained system showed highly selective targeting and

destructive effects on both the cancer cells and xenograft solid tumors implanted in mice under NIR laser irradiation, leading, in some of the treated animals, to the complete disappearance of the tumors.

More recently, various therapeutic techniques have been combined to enhance treatments efficacy, often taking advantages from the increasing number of surface conjugation strategies. PDT therapy for example, which require the use of a photosensitizer molecule able to generate the toxic singlet oxygen specie, has started to be coupled with PTT, due to the possibility to conjugate these photosensitizer agents onto the surface of GNRs. Wang *et al*.[108] conjugated Rose Bengal (RB) molecules onto GNRs: the so-obtained GNRs-RB system exhibited efficient singlet oxygen generation when illuminated by 532 nm green light, due to the presence of RB, and can be used for PTT at 810 nm NIR irradiation, due to the presence of GNRs, thus presenting two different mechanisms for cancer cell death. *In vitro* tests showed also that RB could improve the uptake of GNRs by cancer cells. *In vivo* experiments onto hamster cheek pouches (a model for human oral cancer) demonstrated that combined PDT-PTT capabilities provide better therapeutic effects against oral cancer in comparison to single strategy treatments.

With a similar strategy, Terentyuk *et al*,[109] fabricated GNRs with a silica shell hematoporphyrin (HP)- doped, which can be used for combining PDT and PTT *in vivo*, since they presented absorbance peaks at both 633 nm (HP) and 820 nm (GNRs). Large solid tumors *in vivo* in xenograft tumor rat model were treated after intratumor injection and simultaneous irradiation at the two reported wavelengths. Moreover the efficiency of the combined therapy was evaluated by OCT, demonstrating perfectly the concept of theranostic approach. Tumor volume was also monitored during a 21-day period: the combined PDT and PTT treatments resulted in the large-area tumor necrosis and led to dramatic decrease in the tumor volume.

The same strategy is at the base of the work proposed by Wang *et al*.[66] and already described in the paragraph regarding oligonucleotides conjugation: indeed the system consisting in the aptamer switch probe (ASP) linking the photosensitizer molecule chlorin e6 conjugated onto the surface of GNRs, was exploited both to target cancer cells, for PDT and for PTT, showing once again that combining various strategies is the key for a real improvement of the therapeutic effect.

Finally, as other example of theranostic, Jang *et al*.,[110] developed a GNRs-photosensitizer complex for NIR fluorescence imaging and PTT/PDT cancer therapy (**Figure 12**).

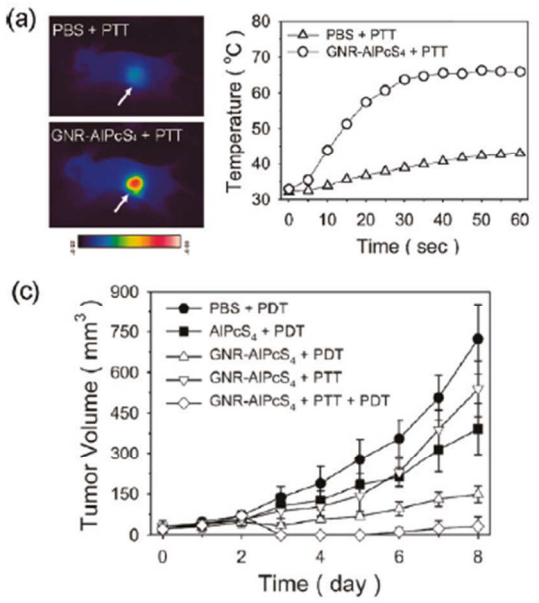

Figure 12 combined therapy and theranostic application of GNRs photosensitizer system. Adapted from B. Jang, *ACS Nano*, 2011, **5**, 1086.

This system resulted non-toxic while in circulation, due to the quenched fluorescence emission and singlet oxygen generation by the photosensitizer close to the GNRs surface, but it became strongly toxic once in cancer cells where the photosensitizer can be detached from the metallic surface. Thus, after intravenous injection of the GNRs complex, the tumor sites were clearly identified on NIR fluorescence images, and PDT can be activated as well as PTT. Tumor growth reduced by 95% with dual PTT and PDT demonstrating the wonderful possibilities to treat effectively and with minimum side effects several cancer types.

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REVIEW

Table 2: summary of surface modified GNRs application and remarkable results.

Entry	Surface modification	Imaging	Therapy	Target	Remarkable results	ref
1	anti-EGFR monoclonal antibodies	DFM	PTT	*in vitro*: epithelial cell line HaCat (non-malignant) HOC313 clone8, HSC3 (malignant).	Twice of uptake in malignat cells.	48
2	PSS and anti-EGFR monoclonal antibody	TPL	/	*in vitro:* A431 skin cancer cells.	TPL intensity from cells with GNRs 3 orders of magnitude brighter than the one from cells alone.	75
3	mPEG-SH and Matrigel	PT-OCT	/	*in vivo:* healthy mice - ear blood vessel.	imaging possible at depths approaching 1 mm *in vivo*.	91
4	polyacrylic acid and anti-Her-2/neu monoclonal antibody	PA	/	*in vitro:* LNCaP prostate cancer lines.	GNRs-antibody concentration in cells an order of magnitude higher than GNRs alone	94
5	HER2 and CXCR4 monoclonal antibodies	PA	/	*in vitro:* MBT2 (murine bladder cancer) and HepG2 (Human epathoma) cell lines.	PA signals enhanced by 7–12 dB and good correlation to specific bindings.	95
6	mPEG-SH or PSS	PA	/	*in vivo:* nude mice.	possibility to detect and localize GNRs at low concentration deep within tissue.	96
7	organic thiol ligand (figure 5e), PLGA-b-PEG and Chlorotoxin	PA	/	*in vitro:* Balb/3T3 mouse fibroblasts.	optical detectability of GNRs at 11 µM with no-cytotoxicity below 20 µM.	27
8	anti-HER2 and anti-EFGR monoclonal antibodies	PA	/	*in vitro*: oral cancer OECM1 and Cal27. *in vivo*: subcutaneous-tumor bearing mice.	imaging with multiple selective targeting demonstrated.	97
9	PSS, PHA and PLA	US	PTT	*in vitro:* HeLa cells. *in vivo:* rabbit kidney.	low toxicity, *in vivo* high resolution imaging possibility and PTT on cells	98
10	mPEG-SH and silica	PAUS	PTT	*in vivo*: epithelial subcutaneous-tumor bearing mice.	PAUS images acquired continuously during PTT. 53°C reached in tumor.	101
11	indocyanine green and silica	X-ray CT NIR fluorescence	/	*in vivo:* gastric cancer subcutaneous-tumor bearing mice	dual mode imaging capability of a single nanoparticle probe using CT and NIR fluorescence.	102
12	silica and folic acid	X-ray CT	PTT	*in vitro:* MGC803 gastric cancer cells. *in vivo:* gastric cancer subcutaneous-tumor bearing mice.	three times higher uptake with folic acid. Excellent PTT effects on cells and strong X-ray attenuation for in vivo X-ray CT imaging.	22
13	organic thiol ligand (figure 5e), PLGA-b-PEG and Chlorotoxin	PA	PTT	*in vitro:* U87MG glioblastoma cells. *in vivo:* glioblastoma cancer subcutaneous tumor bearing mice.	enhanced binding affinity toward GBM cells. Cells damage after laser irradiation. Higher tumor retention with targeted GNRs.	80
14	transferrin and PEG	TPL	PTT	*in vitro:* HeLa cells.	pronounced difference of the cellular uptakes between targeted and non-targeted GNRs	107
15	mPEG-SH	/	PTT	*in vivo:* squamous cell carcinoma xenograft tumor bearing mice.	accumulation in tumor due to EPR effect. Selective hyperthermia of malignant tissues reduced tumor growth.	81
16	PAMAM dendrimers and RGD peptide	/	PTT	*in vitro:* A375 melanoma cells. *in vivo*: xenograft tumor bearing mice.	highly selective targeting and destructive effects. Disappearance of the tumor.	26
17	PAH and RB molecules	/	PTT PDT	*in vitro:* Cal-27 human oral squamous cell	improved photodynamic efficacy due to enhanced uptake of RB by	108

				carcinoma cell line. *in vivo:* hamster cheek pouches.	cancer cells.	
18	silica and hematoporphirin	OCT	PTT PDT	*in vivo:* Alveolaris liver cancer xenograft tumor rat model.	large area of tumor necrosis and decrease in tumor volume.	109
19	mPEG-SH, ASP and Ce6 photosensitizer	/	PTT PDT	*in vitro:* CCRF-CEM acute lymphoblastic leukaemia cell line.	high specific internalization by the target cancer cells. Selective PTT and PDT upon laser irradiation.	66
20	mPEG-SH, RRLAC peptide and AlPcS4 photosensitizer	NIR fluorescence	PTT PDT	*in vitro:* SCC7 squamous cell carcinoma. *in vivo:* SCC7 cancer xenograft tumor bearing mice	intracellular uptake of AlPcS4 improved by about 4-fold. Highly effective PTT/PDT dual therapy proved *in vivo*.	110

5. Conclusion

There are plenty of fascinating features in the surface's properties of GNRs and in this review we have demonstrated the tremendous advantages that can be achieved by using surface modified GNRs for nanomedicine applications. Several of the most important achieved results, relied in a well-fashioned, highly-selective and specifically-designed surface chemistry modifications of these nanostructures. Without efforts in discovering and engineering always novel surface coating mostly of these outcomes should have not be possible. Scientific literature concerning this filed is increasing enormously in the last years and GNRs are continuing to gain interest with every year that passes. Imaging and therapy are clearly clinically transferable with GNRs and for this reason surface engineered GNRs can became the weapon of election in the next years to treat cancer through the theranostic approach.

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Notes and references

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